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ORIGINAL ARTICLE

Effects of altered expression of *LEAFY COTYLEDON1* and *FUSCA3* on microspore-derived embryogenesis of *Brassica napus* L.

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Oil content

Abstract *Brassica napus* (*Bn*) microspore-derived embryogenesis has become a model system to study basic aspects of plant development. Recognized transcription factors governing embryogenesis include: *FUSCA3* (*FUS3*), a member of the plant-specific B3-domain family, and *LEAFY COTYLEDON1* (*LEC1*), a member of the HAP3 subunit of the CCAAT binding factor family. The effects of altered expression of both genes were investigated during microspore-derived embryogenesis in established *B. napus* lines over-expressing or down-regulating *BnLEC1*, as well as in tilling lines where *BnFUS3* was mutated. While over-expression of *BnLEC1* decreases the yield of microspore-derived embryos (MDEs) without affecting their ability to regenerate plants, suppression of *BnLEC1* or *BnFUS3* reduced both embryo number and regeneration frequency. Embryos produced by these lines showed structural abnormalities accompanied by alterations in the expression of several embryogenesis-marker genes. Oil accumulation was also altered in the transgenic MDEs. Total oil content was increased in MDEs over-expressing *BnLEC1* and decreased in those suppressing *BnLEC1* or *BnFUS3*. Mutation of *BnFUS3* also resulted in a small but significant increase in linoleic (C18:2) acid. Together this study demonstrates the crucial role of *BnLEC1* and *BnFUS3* during *in vitro* embryogenesis.

1. Introduction

Embryogenesis is one of the most important events in the life cycle of plants. The process begins with the double fertilization marking the formation of the zygote and the endosperm. During embryogenesis, the zygote divides producing characteristic embryogenic stages (globular, heart-shaped, and torpedo-shaped) that are accompanied by profound molecular, physiological, and metabolic changes [55]. During the middle-late stages of embryogenesis, the embryos accumulate storage products and undergo desiccation prior to entering a dormant period [20,3]. Most of these events are also observed during *in vitro* embryogenesis, where embryos can be produced without fertilization. *In vitro* produced embryos proceed through a similar developmental pathway characteristic of seed embryos and are therefore utilized as a model system [37]. Studies using

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in vitro embryos have some advantages: the embryos develop in the absence of maternal tissue, can be produced in high numbers and in a synchronous fashion, and can be easily harvested. Due to the development and optimization of propagation protocols [17,49], investigations on *in vitro* plant embryogenesis have grown exponentially over the past few years.

In vitro embryogenesis can be executed through different methods, with gametophytic embryogenesis being routinely used in many species. Gametophytic embryogenesis involves the utilization of microspores (or immature pollen grains) and precise culture treatments to induce embryo formation, i.e. microspore-derived embryos (MDEs, Fig. 1). The process uses several types of stress which repress the gametophytic pathway in favor of the embryogenic pathway [9,26,36,41,48,54,57].

Microspore-derived embryogenesis is largely used to propagate *Brassica napus* L. (canola), an economically important species used for oil production. Oilseed rape (canola/rapeseed) oil is the third most important vegetable oil in the world [52]. The production of canola oil relies on the genetic potential of canola cultivars to produce high seed yield and high seed oil content. The quality of canola seed oil is determined by the fatty acid (FA) composition. The process of FA biosynthesis during seed maturation is genetically controlled, and requires the synchronization of several biochemical pathways. Fatty acids and triacylglycerols (TAGs) accumulate during embryo and seed maturation [1,5], making this stage crucial when attempting to increase seed oil content. Independent studies have shown that FA biosynthesis is controlled by the expression of several transcription factors, including *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), *FUSCA3* (*FUS3*), *WRINKLED1* (*WRI1*), and *ABSCISIC ACID INSENSITIVE3* (*ABI3*), which interact to regulate different phases of embryo development and seed maturation [18,32,46]. Many of these genes have critical roles during embryo development [29]. *LEC1* is expressed throughout the entire process of embryogenesis, from the initial to the late developmental stages [56], while *FUS3* is responsible for inducing the maturation phase [8,39]. The *LEC1* protein has the HAP3 subunit of the CCAAT binding factor that allows *LEC1* to be a specific transcriptional regulator of downstream genes containing the CCAAT recognition domain.

FUS3, encoding a B3 protein that accumulates mainly during seed maturation, binds to the RY element CATGCA found in the promoters of several genes [8,39]. Current literature indicates that ectopic expression of *LEC1* is sufficient to induce somatic embryogenesis from vegetative tissue; thus, suggesting a role in regulation of embryogenic competence [29,46,5]. *Arabidopsis* plants with a null *lec1* allele produced abnormal embryos characterized by small hypocotyls and cotyledons [32]. *Arabidopsis fus3* and *lec1* mutant plants also show a decrease in protein and lipid accumulation during seed development [32,21]. As synthesis and storage of oil is linked to several stages of embryo and seed development, it has been suggested that the genetic regulation of embryo morphogenesis and maturation influences oil production. In our previous studies, we demonstrated that over-expression of *BnLEC1* increases seed oil accumulation in *B. napus*, while suppression of *BnLEC1* or *BnFUS3* decreases oil content [11,12]. While these studies suggest a clear involvement of these genes during *in vivo* embryogenesis, little information is available regarding *in vitro* embryogenesis.

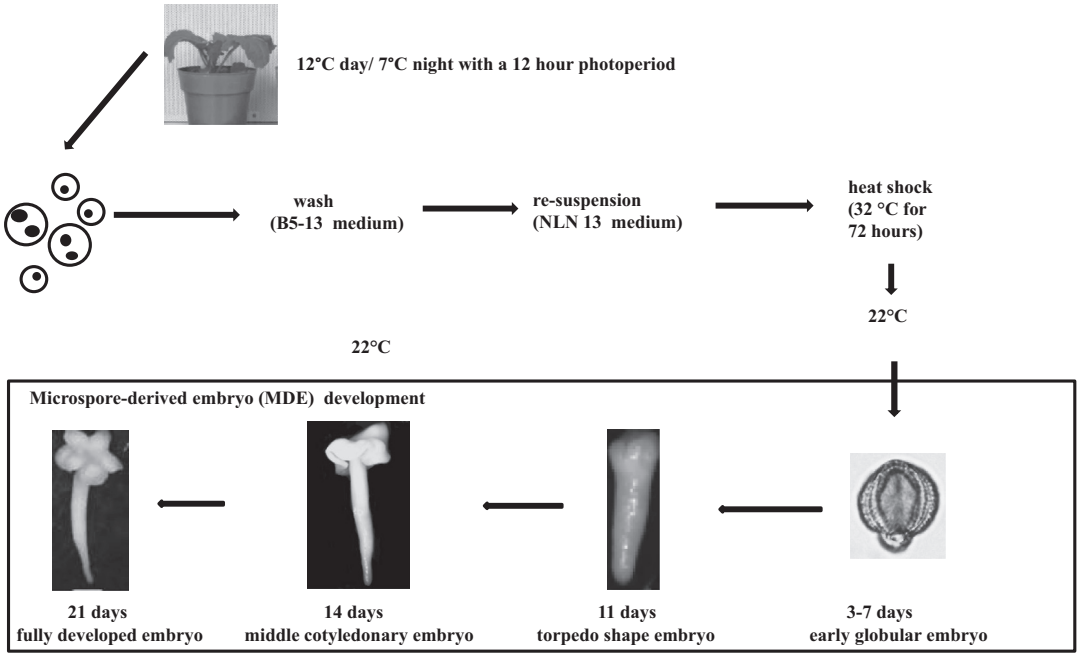

Figure 1 Schematic diagram of microspore-derived embryo development in *Brassica napus*. Plants with young buds were grown in cabinets set at 12 °C day/7 °C night with a 12 h photoperiod. Flower buds (2-3 mm in length) were harvested, sterilized, and ground in a mortar with half strength B5-13 medium supplemented with 13% (w/v) sucrose. The homogenate was centrifuged at 750 rpm (g) at 4 °C for 3 min and this process was repeated three times. The microspore-containing pellet was thereafter re-suspended and diluted in NLN-13 medium with 13% sucrose (pH 5.8) to a concentration of 10,000 microspores/ml. Embryo development was triggered after an initial heat shock treatment at 32 °C for 72 h. Embryos were subsequently incubated at 22 °C on a shaker set at 80 rpm. The number below each developmental stage of MDEs shows the days in culture.

Assessing whether oil production and fatty acid composition during *in vitro* embryogenesis are under the control of similar genes and regulatory mechanisms operating during *in vivo* embryogenesis could have significant applications. This discovery would allow plant scientists to utilize the *in vitro* embryogenesis system as a model to study oil biosynthesis, as well as rapidly screen for desirable oil-related traits or manipulate oil production in culture prior to plant regeneration. This knowledge could save time and resources and has the potential to make significant advances in plant-based oils research. Another important factor to consider when exploiting the advantages of *in vitro* embryogenic systems, is whether the introduction of the desirable trait has pleiotropic effects compromising the number of embryos produced and their quality, i.e. their ability to regenerate viable plants, as both are key parameters for propagation.

In line with these considerations, the purpose of the present study is to determine how altered expression of *BnLEC1* and *BnFUS3* influences *B. napus* microspore-derived embryogenesis, with emphasis on morphological characteristics determining embryo quantity and quality, as well as oil accumulation and fatty acid profile. The results obtained will then be compared to previous studies to assess similarities or differences between *in vivo* and *in vitro* systems.

2. Materials and methods

2.1. Plant material

Transgenic canola plants over-expressing (*B. napus* var. Polo) or down-regulating (*B. napus* var. Topas) *BnLEC1,* as well as tilling mutant lines (*B. napus* var. DH12075) suppressing *BnFUS3* were generated and characterized in previous studies [11,12]. Two versions of *BnLEC1* were used in those studies: version A (GU945399) and version B (GU945398), which differ by 13 nucleotides [Supplementary Fig. 1 in 12] and 4 amino acids [Supplementary Fig. 2 in 12]. For the present work we used two lines over-expressing version A of *BnLEC*1 (lines S1 and S2), two lines over-expressing version B of *BnLEC*1 (lines S3 and S4), and two lines down-regulating version B (A1, and A2). Given the high similarity in nucleotide sequence of the two versions, anti-sensing version A also suppressed version B [12].

Three *B. napus BnFUSCA3* tilling mutant lines [M1-3] with point mutations changing one amino acid downstream of the B-3 DNA binding domain of *FUS3* were generated and characterized in previous work [11].

2.2. Generation of B. napus microspore-derived embryos (MDEs)

BnLEC1 transgenic lines and *BnFUS3* tilling mutant lines were used as the source of microspores to generate microspore-derived embryos (MDEs), following the procedure of Belmonte et al. [2]. Plants with young buds were grown in a cabinet set at 12 °C day/7 °C night with a 12 h photoperiod. Flower buds (2–3 mm in length) were harvested, sterilized in 10% bleach and ground in a mortar in half strength B5-13 medium supplemented with 13% (w/v) sucrose. The homogenate was centrifuged at 750 rpm (g) at 4 °C for 3 min. The microspore-containing pellet was thereafter re-suspended in NLN-13 medium with 13% sucrose (pH 5.8) and further diluted in NLN-13 medium to a concentration of 10,000 microspores/ml. Embryo development was triggered after an initial heat shock treatment at 32 °C for 72 h followed by incubation at 22 °C on a shaker set at 80 rpm. The number of microspore-derived embryos was counted after 21 d in culture [2].

2.3. Determination of microspore-derived embryo quality

Embryo quality was assessed by the ability of the MDEs to regenerate viable root and shoot systems at germination. Fully mature (21 d) embryos were germinated on half concentration Murashige and Skoog (MS) medium [35] and the number of seedlings with fully developed shoots and roots were scored, as reported [2].

2.4. Microscopy

For histological examinations, fully mature embryos were fixed in 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05 M phosphate buffer (pH 6.9), dehydrated with methyl cellosolve followed by three washings with absolute ethanol, and then infiltrated and embedded in Historesin (Leica, Concord, Ontario, Canada). Sections (3 μm) were stained with periodic acid-Schiff (PAS) reagent and then counterstained with toluidine blue (TBO) according to the methods of Yeung [59].

2.5. Determination of lipid profile and fatty acid (FA) composition

All analyses were conducted in the University of Manitoba seed quality lab, which is certified annually by the Canadian Grain Commission. Total lipid contents were quantified using the modified Swedish method as described by Troëng, [50]. Fatty acid (FA) composition analyses were conducted using gas chromatography (Varian, Walnut Creek, USA) as documented by Hougen and Bodo [23]. In short, approximately 5 g of mature *B. napus* MDEs were dried overnight at 40 °C. Then, 1 g of dried MDEs was crushed and homogenized in 5 ml of heptane and incubated at room temperature for 24 h. The supernatants were then poured into clean 13 × 100 test tubes and after addition of 500 μl of 0.5 N sodium methoxide, the samples were shaken for 30 min. 100 μl of acidified water (0.3% acetic acid) was added to the samples which were then incubated at 4 °C for 2 h. Lastly, 500 μl of reaction mixture was poured into a 2 ml auto sampler (Fisher brand vial CAT# 03-391-16) [7,27]. Gas chromatography was performed using a Varian model 3900 fitted with a CP-Wax 52 CB capillary column and a flame ionization detector. Peak areas were measured by the Varian Star Workstation software system. A reference standard, GLC # 421, bought from Nu-Check Prep (Elysian, Minnesota) was used to confirm the appropriate GC process.

2.6. Gene expression analysis by quantitative qRT-PCR

Analysis of gene expression in developing [7,14, and 21 day] *B. napus* MDEs was determined by quantitative qRT-PCR [13]. Expression studies were conducted for molecular marker genes identified in previous studies [14,31] and required for proper embryo formation.

These genes are classified as embryo specific; *LEAFY COTYLEDON2* (*LEC2*), UKNOWN *PROTEIN1* (*UPI1*); embryo expressed *BABYBOOM1* (*BBM1*), *WUSCHEL-related HOMEOBOX9* (*BnWOX9*), *ABSCISIC ACID3* (*ABI3*) and sporophyte expressed *CYTOCHROME P78A* (*CYP78A*), and *WUSCHEL-related HOMEOBOX2* (*BnWOX2*).

These analyses also included genes involved in glycolysis: *FRUCTOSE BISPHOSPHATE ALDOLASE* (*BnFPA*), *PHOSPHOGLYCERATE KINASE* (*BnPGK*), *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), *HEXOSE KINASE* (*BnHXK*) and *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* (*BnPPK*); sucrose transport and metabolism: *SUCROSE TRANSPORTER 1* and *4* (*BnSUC 1* and *4*), *SUCROSE SYNTHASE 1* and *3*, (BnSUS1 and 3), *ADP-GLUCOSE PHOSPHORYLASE* (*BnAGP*); and FA biosynthesis: *SUBUNIT A* of *ACETYL-CoA CARBOXYLASE* (*BnACCA2*), *ω-3 FA DESATURASE* (*BnFAD3*), *FA ELONGATION1* (*BnFAE1*), and *MALONYL-CoA:ACP TRANSACYLASE* (*BnMCAT*). The relative level of gene expression was determined with the $2^{-\Delta\Delta}$CT method [28] using UBC21 (*EV086936, ubiquitin-conjugating enzyme 21*) as a reference. All PCR reactions were performed using the CFX96™ Optics Real- Time System (Bio-Rad, Hercules, CA) with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s (denaturation), and 59 °C for 10 s (annealing/extension). The melting curve rose from 65 °C to 95 °C by increments of 0.5 °C every 5 s.

Analyses were performed on three biological replicates. All primers used are listed in Supplementary Tables 1 and 2.

2.7. Statistical analysis

The statistical analyses were performed using the SAS® 9.3 program (SAS Institute Inc., Cary, NC, USA.). Unless specified, all experiments were performed using at least three biological replicates and the Fisher's Least Significant Difference (LSD) test was applied to compare samples.

3. Results

Plants from the lines utilized have been fully characterized in previous studies [11,12]. Of the four *BnLEC1* over-expressing lines utilized, two (S1 and S2) over-expressed version A of *BnLEC1*, and two (S3 and S4) over-expressed version B. Expression of *BnLEC1* was induced in both vegetative and reproductive tissue of the four lines [Supplemental Fig. 3 in 12], as well as during the different phases (7, 14, and 21 day) of microspore-derived embryogenesis (Fig. 2). Lines with anti-sense *BnLEC1* (A1 and A2) had reduced expression of *BnLEC1* in the same tissues [Supplemental Fig. 3 in 12] and during microspore-derived embryogenesis (Fig. 2). The *BnFUS3* lines (M1-3) had point mutations resulting in a non-functional *BnFUS3* protein [11].

3.1. Effects of altered expression of BnLEC1 and BnFUS3 on microspore-derived embryo quantity and quality

The *BnLEC1* transgenic lines and *BnFUS3* mutant lines were utilized as the source of microspores to produce embryos in culture. Compared to the WT, the number of fully developed (day 21) MDEs produced by lines over-expressing version A (S1, S2) or version B (S3, S4) *BnLEC1*, as well as by lines down-regulating *BnLEC1* (A1, A2) were significantly repressed (Fig. 3A and B). With the exception of line S1, embryo formation was less than half in all *BnLEC1* transgenic lines. A similar and consistent repression in embryo number was also observed in the three lines (M1-3) where *BnFUS3* was mutated (Fig. 3C).

The quality of MDEs, i.e. their ability to regenerate viable plants at germination, was not affected by the over-expression of *BnLEC1* (S1-4), while it decreased in those where the expression of *BnLEC1* was suppressed (A1, A2), and in the *BnFUS3* mutant lines (M1-3) (Fig. 4).

3.2. Effects of altered expression of BnLEC1 and BnFUS3 on embryo morphology

Microspore-derived embryo morphology was assessed at day 14, corresponding to the cotyledonary stage of development and in fully developed embryos (day 21). At both days identical phenotypes were observed within lines characterized by similar expression of the transgene.

After 14 d in culture, MDEs over-expressing *BnLEC1* (S1-4) had a similar morphology to their WT embryos and consisted of an elongated embryonic axis and fully expanded cotyledons (Fig. 5A(a–b)). This was in contrast to MDEs suppressing *BnLEC1* (A1-2) where the elongation of the embryonic axis was inhibited and the proper formation of cotyledons was compromised (Fig. 5A(c)). Embryos generated from the *BnFUS3* mutant lines (M1-3) shared similar abnormalities including embryonic axes characterized by unorganized cell proliferation (Fig. 5A(d)). The cotyledons of these embryos were also generally larger and partially fused (Fig. 5A(d)).

The phenotypic abnormalities observed at 14 d were retained by fully developed (21 d) MDEs (Fig. 5A(e–h)). The only exception was observed in embryos produced by the *BnLEC1* down-regulating lines (A1-2) which showed partially fused embryonic axes and abnormal cotyledons (Fig. 5A(g)).

The most obvious morphological abnormalities were also analyzed at a structural level in relation to lack of a shoot apical meristem observed in the *BnLEC1* down-regulating embryos relative to WT embryos (Fig. 5B(a,b)), and the presence of irregular outgrowth along the hypocotyl of BnFUS3 tilling mutant embryos (Fig. 5B(c)).

3.3. Expression profiles of selected molecular markers of microspore-derived embryogenesis

To better understand the role of altered *BnLEC1* and *BnFUS3* expression during embryogenesis and the morphological abnormalities resulting from their miss-expression, we also measured the transcript abundance of 8 molecular marker genes at different stages of MDE development (late-globular, 7 day; cotyledonary, 14 day; and fully developed, 21 day). Expression of these genes was linked to proper embryonic development [14,31].

An overall increase in transcript levels of *BnLEC2* and *BnAB13* was observed in late-globular (7 day) MDEs of the *BnLEC1* over-expressing lines (S1–4) (Fig. 6). The expression

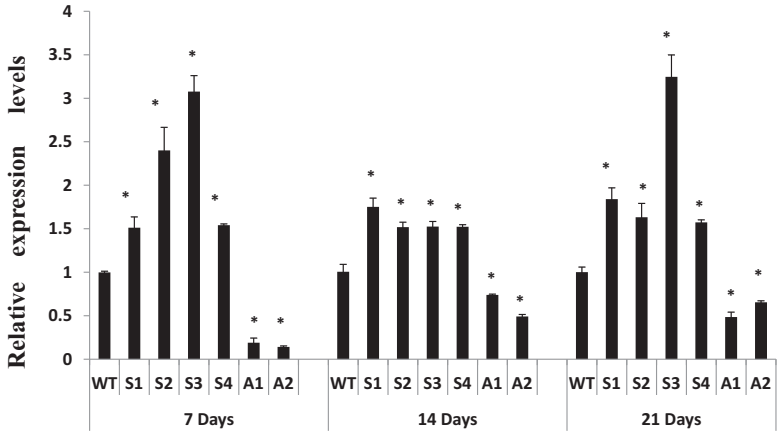

Figure 2 Expression analysis by quantitative (q)RT-PCR of *LEAFY COTYLEDON1* (*LEC1*) in transgenic *BnLEC1* microspore-derived embryos at different days in culture. Data presented are mean value ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the wild type value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1–S4), *BnLEC1* down-regulators (A1–A2).

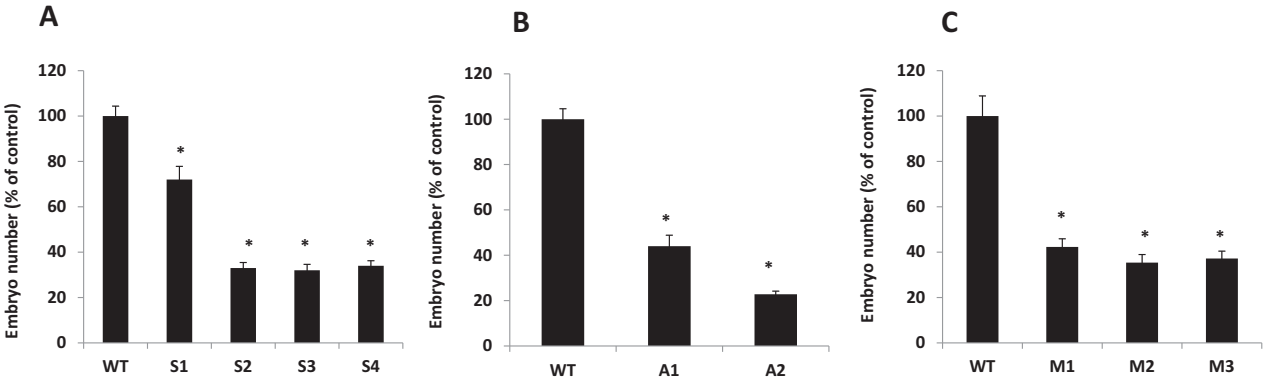

Figure 3 Production of microspore-derived embryos (MDEs) from *Brassica napus* plants with altered expression of *BnLEC1* (A,B) and *BnFUS3* (C). *BnLEC1*over-expressing lines (S1–S4), *BnLEC1* down-regulating lines (A1–A2) and *BnFUS3* tilling mutant lines (M1–M3) were utilized to generate MDEs. For each experiment, 100,000 microspores were plated and embryo number was counted at 21 d. Value ± SE ($n = 5$) is expressed as a percentage of the WT. Asterisks indicate values that are statistically different (LSD: $P < 0.05$) from the respective WT (control) value (set at 100).

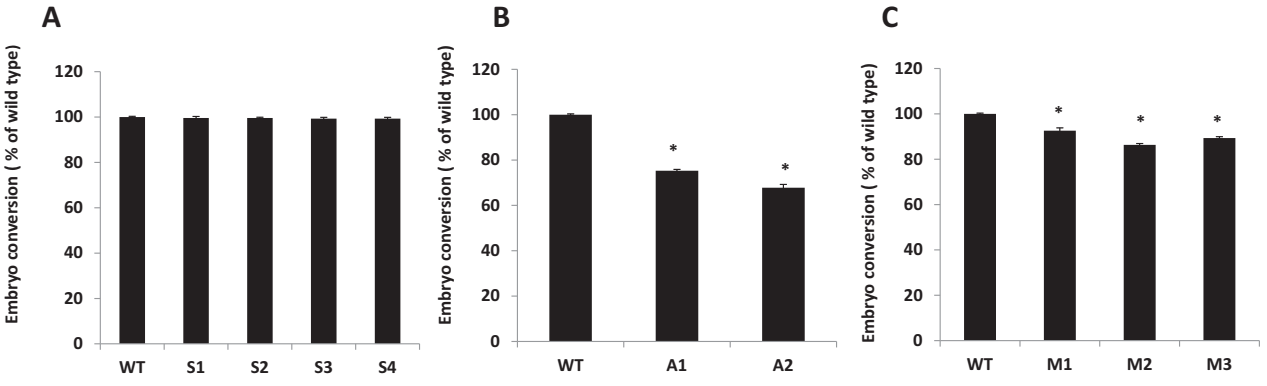

Figure 4 Conversion frequency (ability to form viable shoots and roots at germination) of MDEs with altered expression of *BnLEC1* (A, B) and *BnFUS3* (C). *BnLEC1* over-expressing lines (S1–S4), *BnLEC1* down-regulating lines (A1–A2) and *BnFUS3* tilling mutant lines (M1–M3) were utilized to generate MDEs. Value ± SE ($n = 3$) is expressed as a percentage of the WT. Asterisks indicate values that are statistically different (LSD: $P < 0.05$) from the respective WT value (set at 100).

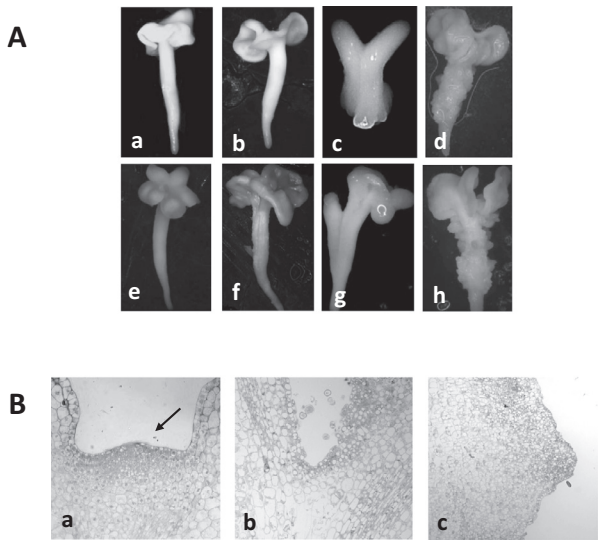

Figure 5 Effects of altered expressions of *BnLEC1* and *BnFUS3* on morphology of microspore-derived embryos (MDEs). (A) Morphology of 14 and 21- day old (early and late cotyledonary stages) MDEs. Early cotyledonary stages of MDEs (a–d); (a) wild type, (b) S4, (c) A1, (d) M1. Late cotyledonary stages of MDEs (e–h); (e) wild type, (f) S4, (g) A1, (h) M1. (B) Effects of altered expression of *BnLEC1* and *BnFUS3* on the structure of *B. napus* MDEs after 21 days in culture. (a) Shoot apical meristem of wild type (WT) MDEs. The meristem was dome-shaped and composed of many layers of densely cytoplasmic cells (arrow), (b) Poorly-developed meristem of MDEs down-regulating *BnLEC1* (line A1). (c) Irregular outgrowth along the epidermal layer of the hypocotyl of MDEs *BnFUS3* tilling mutant M3.

of the other genes at the same stage of development did not follow any consistent profile. At 21 d, over-expression of *BnLEC1* resulted in a significant induction of *BnLEC2* and *BnCYP78A5* in all lines (S1–S4), as well as *BnUPI1*, *BnWOX2*, and *BnWOX9* in lines S1–S3 and *BnSERK1* in lines S2–S4. A reduction in the transcript levels of *BnAB13* was observed in fully developed embryos of lines S1-S3.

In 7 day MDEs suppressing *BnLEC1* (A1, A2) an overall repression in transcript levels was measured for many of the genes analyzed including *BnCYP78A5*, *BnWOX2*, *BnLEC2*, *BnUPI1*, *BnBBM1*, and *BnABI3* (Fig. 6). This was in contrast to the expression of *BnWOX9*, which was highly induced in both *BnLEC1* antisense embryos. Upon further development (day 14), the transcript levels of *BnWOX2*, *BnUPI1* and *BnCYP78A5* increased in both lines. The expression of *BnSERK1* was repressed in fully developed (day 21) *BnLEC1*-suppressing MDEs and this was in contrast to *BnWOX2*, which was induced in both A1 and A2 lines.

In immature (day 7) embryos produced by the *BnFUS3* mutant lines, the only gene showing a consistent expression pattern was *BnWOX9*, which was repressed in all three (M1–3) lines. At day 14, the expression of *BnWOX2* and *BnBBM1* was significantly induced, while that of *BnSERK1* was repressed. Fully developed (day 21) *BnFUS3* mutant MDEs were characterized by increased transcript levels of *BnABI3* and *BnWOX2* and suppression of *BnUPI1* (Fig. 6).

3.4. Effects of altered expression of BnLEC1 and BnFUSCA3 on total lipid and fatty acid accumulation in microspore-derived embryos

The late phases of embryo development are characterized by the accumulation of storage products, which in *B. napus* consist mainly of oil. Previous studies have shown that altered expression of both *BnLEC1* and *BnFUS3* influences oil level and FA composition in seeds [11,12]. To assess if similar changes also occur during *in vitro* embryogenesis, lipid content and FA composition were analyzed in the MDE lines.

Relative to the WT, the lipid content increased in embryos over-expressing *BnLEC1* (S1–S3), while it was reduced in those suppressing *BnLEC1* (A1) and in those where *BnFUS3* was mutated (M1, M2) (Fig. 7).

Analysis of FA composition revealed small alterations in the levels of stearic acid (18:0), and oleic acid (C18:1) in the M2 embryos, as well as an increase in linolenic acid (C18:3) content in S3 embryos and linoleic acid (C18:2) in M1and M2 embryos (Table 1).

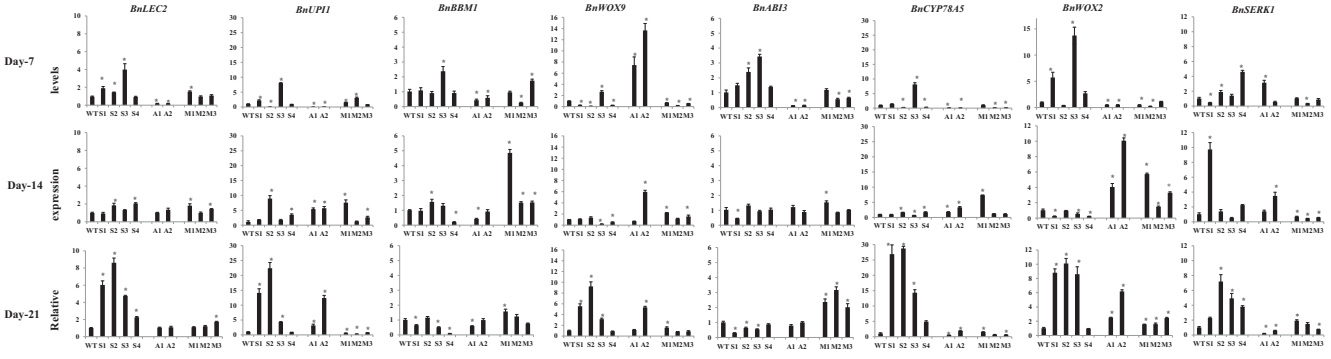

Figure 6 Expression analysis by quantitative (q)RT-PCR of embryogenesis-marker genes in MDEs with altered levels of *BnLEC1* and *BnFUS3*. Analyses were conducted at day 7, 14, and 21. Genes analyzed: *LEAFY COTYLEDON2* (*LEC2*), *UKNOWN PROTEIN1* (*UPI1*), *BABYBOOM1* (*BBM1*), *WUSCHEL-related HOMEOBOX9* (*BnWOX9*), *ABSCISIC ACID3* (*ABI3*), *CYTOCHROME P8A5* (*CYP78A5*), *WUSCHEL-related HOMEOBOX2* (*BnWOX2*), and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*SERK1*). Data presented are mean value ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective wild type (control) value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1–S4), *BnLEC1* down-regulators (A1–A2), *BnFUS3* tilling mutants (M1–M3).

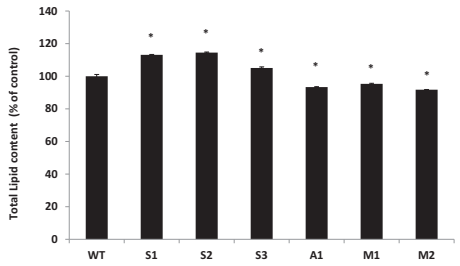

Figure 7 Total lipid content in 21 day-old MDEs with altered expression of *BnLEC1* or *BnFUS3*. Value ± SE ($n = 6$) is expressed as a percentage of the WT. Asterisks indicate values that are statistically different (LSD: $P < 0.05$) from the WT value. *BnLEC1* over-expressors (S1–S3), *BnLEC1* down-regulator (A1), *BnFUS3* tilling mutants (M1–M3).

3.5. Transcriptional regulation of sucrose and fatty acid metabolism genes

The main carbon source for oil biosynthesis in developing seeds is sucrose produced from photosynthesis. Sucrose is readily hydrolyzed into UDP-glucose and fructose, which are oxidized in the glycolytic pathway to Acetyl-CoA, the precursor of FA biosynthesis. The changes in oil levels observed in the transformed MDEs prompted us to measure the transcript levels of genes contributing to sucrose metabolism and glycolysis. At both day 14 and 21 in culture, *BnLEC1* over-expressing MDEs had increased expression levels of several sucrose metabolic genes. These included *SUCROSE SYNTHASE1* and *3* (*BnSUS1* and *3*) at day 14 (Fig. 8) and *SUCROSE TRANSPORTER 4* (*BnSUC4*) and *BnSUS3* at day 21 (Fig. 9). Over-expression of *BnLEC1* also resulted in an increased expression of the glycolytic enzyme *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), as well as a decreased expression of the FA metabolic genes *SUBUNIT A of ACETYL-CoA CARBOXYLASE* (*BnACCA2*) at 14 d. And increased the expression of *MALONYL-CoA:ACP TRANSACYLASE* (*BnMCAT*) at day 21 (Figs. 8 and 9).

An overall suppression of several sucrose and glycolytic enzymes, including *FRUCTOSE BISPHOSPHATE ALDOLASE* (*BnFPA*), *PHOSPHOGLYCERATE KINASE* (*BnPGK*), and *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* (*BnPPK*), as well as several fatty acid metabolic enzymes such as *BnACC2*, *ω-3 FA DESATURASE* (*BnFAD3*), and *FA ELONGATION1* (*BnFAE1*),were observed in MDEs with suppressed level of *BnLEC1* (Figs. 8 and 9).

A similar overall repression in gene expression was also measured in the two *BnFUS3* TILLING mutant MDEs, especially at day 14 (Fig. 8) where the majority of genes encoding enzymes of sucrose and fatty acid metabolism were transcriptionally down-regulated.

4. Discussion

In vitro embryogenesis is an attractive system for studying the molecular and physiological events associated with embryo development [33,38]. Besides producing embryos with similar morphology and developmental stages to their *in vivo* counterparts, *in vitro* embryogenic systems can generate large numbers of synchronized embryos in a short period of time [45]. This is also true for microspore-derived embryogenesis, where the gametophytic fate of the microspores can be redirected toward an embryogenic fate through applications of stress conditions and culture treatments (Fig. 1).

To maximize the use of Brassica *in vitro* embryogenic systems in breeding programs, thus accelerating the selection of desirable traits such as elevated seed oil levels, it is paramount to assess if *in vitro* embryos behave in a similar fashion to their zygotic counterparts in response to gene manipulations. This is especially relevant when examining storage product deposition, a trait controlled by a complicated and partially unknown genetic network [10].

In Brassica seeds *LEC1* and *FUS3* have been identified as key regulators of oil synthesis [11,12]. Oil content is enhanced in seeds over-expressing *BnLEC1*, while repressed in those where the expression of either *BnLEC1* or *BnFUS3* was suppressed [11,12]. To verify if similar alterations occur *in vitro*, we measured total lipid content and FA composition during microspore-derived embryogenesis. Relative to the WT, over-expression of *BnLEC1* increased lipid content in MDEs by about 10–15%, while its down-regulation decreased oil content by 5% (Fig. 7). These values are comparable to those observed *in vivo* (7–16% increase in *BnLEC1* over-expressing seeds and a 9–12% decrease in *BnLEC1* down-regulating seeds) [12]. For both *in vivo* and *in vitro*, these changes were not accompanied

Table 1 Fatty acid profile (% composition) in transgenic *BnLEC1* and *BnFUS3* tilling mutant MDEs. Values are means of three biological replicates ±SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from their respective WT value. S1–S3, *BnLEC1* over-expressors; A1, *BnLEC1* down-regulators; M1–M2, *BnFUS3* tilling mutants.

	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0
WT	5.15 ± 0.25	4.03 ± 0.41	68.44 ± 1.99	13.00 ± 1.59	5.44 ± 0.81	1.15 ± 0.02
S1	5.67 ± 0.37	3.74 ± 0.48	69.48 ± 1.35	12.91 ± 1.41	4.29 ± 0.21	1.09 ± 0.06
S2	5.35 ± 0.25	2.91 ± 0.68	67.08 ± 0.95	14.59 ± 0.23	6.95 ± 0.06	0.94 ± 0.12
S3	5.05 ± 0.25	2.82 ± 0.65	65.88 ± 1.10	15.78 ± 0.97	7.14 ± 0.43*	0.93 ± 0.11
WT	5.86 ± 0.11	2.55 ± 0.19	65.76 ± 0.43	16.86 ± 0.22	6.39 ± 0.82	0.93 ± 0.02
A1	5.78 ± 0.16	2.44 ± 0.24	65.38 ± 0.57	16.85 ± 0.20	6.39 ± 0.77	0.87 ± 0.06
WT	5.57 ± 0.06	2.35 ± 0.19	63.54 ± 1.94	16.33 ± 1.20	7.71 ± 0.18	0.86 ± 0.07
M1	6.01 ± 0.27	3.21 ± 0.49	61.16 ± 2.62	20.10 ± 1.05*	7.12 ± 0.71	1.09 ± 0.15
M2	6.61 ± 0.49	4.09 ± 0.53*	54.88 ± 1.81*	22.51 ± 0.89*	8.58 ± 0.36	1.40 ± 0.23

by any major alterations in FA composition (Table 2 and Supplementary Table 4 in 12, and Table 1 in this paper), but did show major changes in the expression of genes encoding enzymes of sucrose and FA metabolism (Figs. 3 and 4 in 12, and Figs. 8 and 9 in this paper). It is worth noting that both *in vivo* and *in vitro* over-expression of *BnLEC1* induces expression of sucrose metabolic genes as well as genes participating in the glycolytic pathway. This induction denotes an active utilization and oxidation of sucrose which is possibly required for enlarging the pool of FA biosynthetic precursors, leading to the observed increase in seed oil content. This process would be compromised in MDEs down-regulating *BnLEC1*, which like seeds down-regulating *BnLEC1* show a reduction in the expression of several glycolytic enzymes (Figs. 8 and 9). The requirement of *BnLEC1* for oil accumulation, documented in several systems [29,34,42], is associated with an enhancement of carbon flux toward the FA biosynthetic pathway [12,15,34].

Mutation of *BnFUS3* in *B. napus* MDEs reduces oil content and increases the relative level of linoleic (C18:2) acid. A comparable decrease in oil, associated with altered sucrose metabolism and decreased glycolytic activity was also observed in *BnFUS3* mutant seeds [11]. A similar repression of the glycolytic pathway might also occur *in vitro*, as the expression of several glycolytic enzymes is repressed in *BnFUS3* TILLING mutant MDEs (Figs. 8 and 9). Collectively these results show that the effects of *BnLEC1* and *BnFUS3* on oil quantity and composition, as well as on the transcription of sucrose and FA metabolic enzymes, observed in Brassica MDEs mimic closely those reported *in vivo*.

Embryonic yield and quality, i.e. frequency of successful regeneration, are crucial parameters of any propagation system integrated in breeding programs. It was therefore one of the objectives of this study to evaluate if the *BnLEC1* and *BnFUS3* modulation of oil level was accompanied by changes in embryonic response in culture.

LEAFY COTYLEDON1 encodes a HAP3 subunit of the CCAAT-binding transcription factor [29] expressed in immature *Arabidopsis* siliques, zygotic embryo and embryogenic tissue, but not in vegetative tissue [24]. Elevated expression of this gene has also been measured in embryogenic carrot cells and somatic embryos [58], as well as immature maize somatic embryos [60], thus suggesting its participation in embryogenesis-related pathways. During *in vitro* embryogenesis in *B. napus*, *BnLEC1* is required both for the formation of MDEs (Fig. 3B), as well as the ability to regenerate viable plants (Fig. 4B). The poor embryogenic competence observed in microspores suppressing *BnLEC1* (lines A1 and A2) suggests that the proper function of this gene is needed for the g ametophytic–embryogenic transition initiating the process. This observation, supporting previous studies on the ability of *LEC1* to induce embryogenesis in vegetative tissue upon ectopic expression [29], is also confirmed by the miss-expression of several embryogenesis marker genes in the *BnLEC1* down-regulating embryos after 7 days in culture. These include *BnABI3* which is a gene originally identified in ABA signaling [25] and implicated in early embryogeny [16,31], and *BnBBM1,* which when expressed ectopically is sufficient to spontaneously form somatic embryos in *Arabidopsis*, tobacco, and *B. napus* seedlings [4,44].

The requirement of *BnLEC1* for MDE quality (Fig. 4B) is a novel concept suggesting a potential function of this gene in the establishment of the embryo body, specifically the proper development of cotyledons and the elongation of the embryonic axis in immature embryos (Fig. 5). These phenotypic abnormalities can be partially explained by the likely function of *LEC1* downstream of auxin responses [16]. The role of auxin in the establishment of the bilateral symmetry during embryogenesis is well established and interference with the auxin flow results in malformed and partially fused cotyledons and stunted elongation of the embryonic axis [6,51]. These structural abnormalities, apparent in MDEs down-regulating *BnLEC1* (Fig. 5), compromise their regeneration and correlate to the miss-expression of several embryogenesis genes, including the auxin-regulated gene *BnWOX2*. This gene modulates the auxin effects on embryonic tissue patterning and its altered expression compromises the normal progression of embryogenesis [19]. Deviations from the normal embryogenic pathway caused by the suppression of *BnLEC1* might also be caused by the altered expression of other embryo marker genes such as *BnLEC2*, necessary for maintenance of the suspensor and specification of cotyledons [46], as well as *BnCYP78A*, and *BnUPI1*, which have been associated to the normal development of MDEs [31].

Unlike its suppression, over-expression of *BnLEC1* only affects the number of embryos produced (Fig. 3A), but not their morphology and quality (Fig. 4A). Among possible reasons for the interference with the initiation of the embryogenic pathway is the miss-expression of *BnSERK1* at day 7. This gene encodes one of the several kinase regulators of the reprogramming of embryogenesis [22]. Originally characterized in carrot, *SERK1* has been reported in many other plant species, including *Arabidopsis* and wheat where it enhances embryogenic competence *in vitro* [22,40,43].

Embryo quantity and quality were greatly impaired in mutants of *BnFUS3*, a gene encoding a B3 domain-containing protein present in developing embryos from a very early stage to immediately before germination [30]. Together with *LEC1*, *FUS3* is a key regulator of *in vivo* embryogenesis [53]; however, the different morphological defects caused by the suppression of the two genes suggest separate roles. Besides the repressive effect on microspore-derived embryogenesis documented in our study (Fig. 3C), mutations of *FUS3* also impair *Arabidopsis* somatic embryogenesis [16], observations denoting the function of this gene are retained across different species and embryogenic systems. The abnormal behavior observed in the *BnFUS3* mutant lines is associated with profound alterations in the expression of many embryo marker genes. Of interest, are the consistent repression of *BnWOX9* at day 7 and the induction of *BnWOX2* at day 14 and 21 that are required for the establishment of polarity domains within the embryo [19]. Furthermore, the increased expression at day 21 of *BnABI3*, suggests possible alterations in ABA signaling. Abscisic acid accumulates during the middle-late stages of embryogenesis and is required for the correct completion of the embryogenic program [47].

Collectively these studies demonstrate that altered expression of *BnLEC1* and *BnFUS3* affects oil accumulation and composition in cultured embryos. While over-expression of *BnLEC1* favors oil accumulation, suppression of either *BnLEC1* or *BnFUS3* reduces oil level. These changes were comparable to those observed in transgenic seeds. Both embryo yield and quality were also affected by the transgenes with over-expression of *BnLEC1* reducing the number of MDEs produced, while suppression of *BnLEC1* or *BnFUS3*

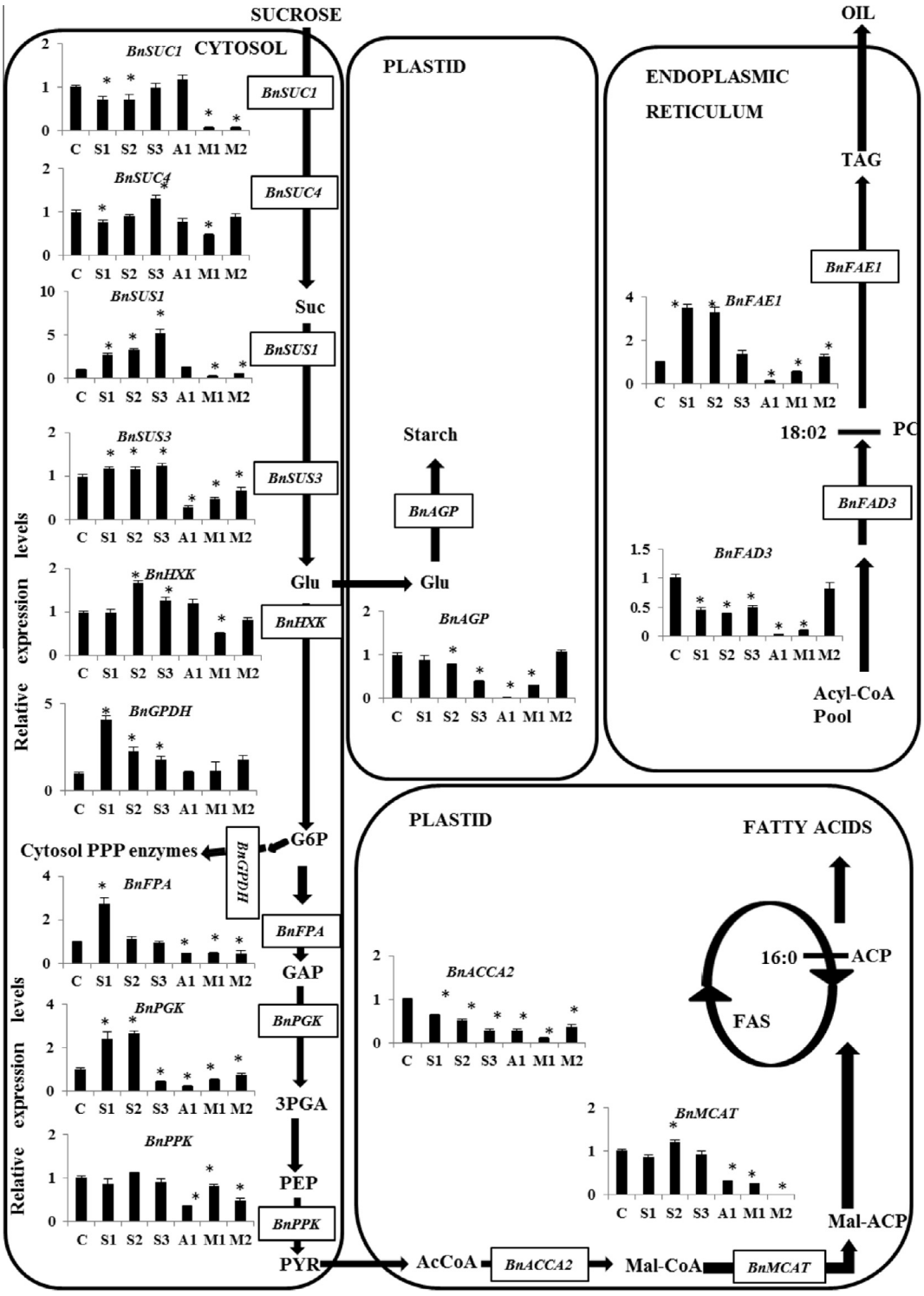

Figure 8 Expression analysis by quantitative (q)RT-PCR of genes involved in the regulatory pathways leading to FA synthesis in *BnLEC1* transgenic and *BnFUS3* tilling mutants MDEs at 14 days. *SUC1* (*Sucrose Transporter1*), *SUC4* (*Sucrose Transporter4*), *SUS1* (*Sucrose Synthase1*), *SUS3* (*Sucrose Synthase3*), *AGP* (*ADP-Glucose Phosphorylase*). *FPA* (*Fructose Bisphosphate Aldolase*), *PGK* (*Phosphoglycerate Kinase*), *GPDH* (*Glyceraldehyde-3- Phosphate Dehydrogenase*), *HXK* (*Hexose Kinase*), *PPK* (*Pyrophosphatase-Dependent Phosphofructokinase*). *ACCA2* (*Subunit A of Acetyl-CoA Carboxylase*), *FAD3* (*ω-3 FA Desaturase*), *FAE1* (*FA Elongation1*), *MCAT* (*Malonyl-CoA:ACP Transacylase*). Data presented are mean value ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective wild type value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1, S3), *BnLEC1* down-regulator (A1), *BnFUS3* tilling mutants (M1, M2).

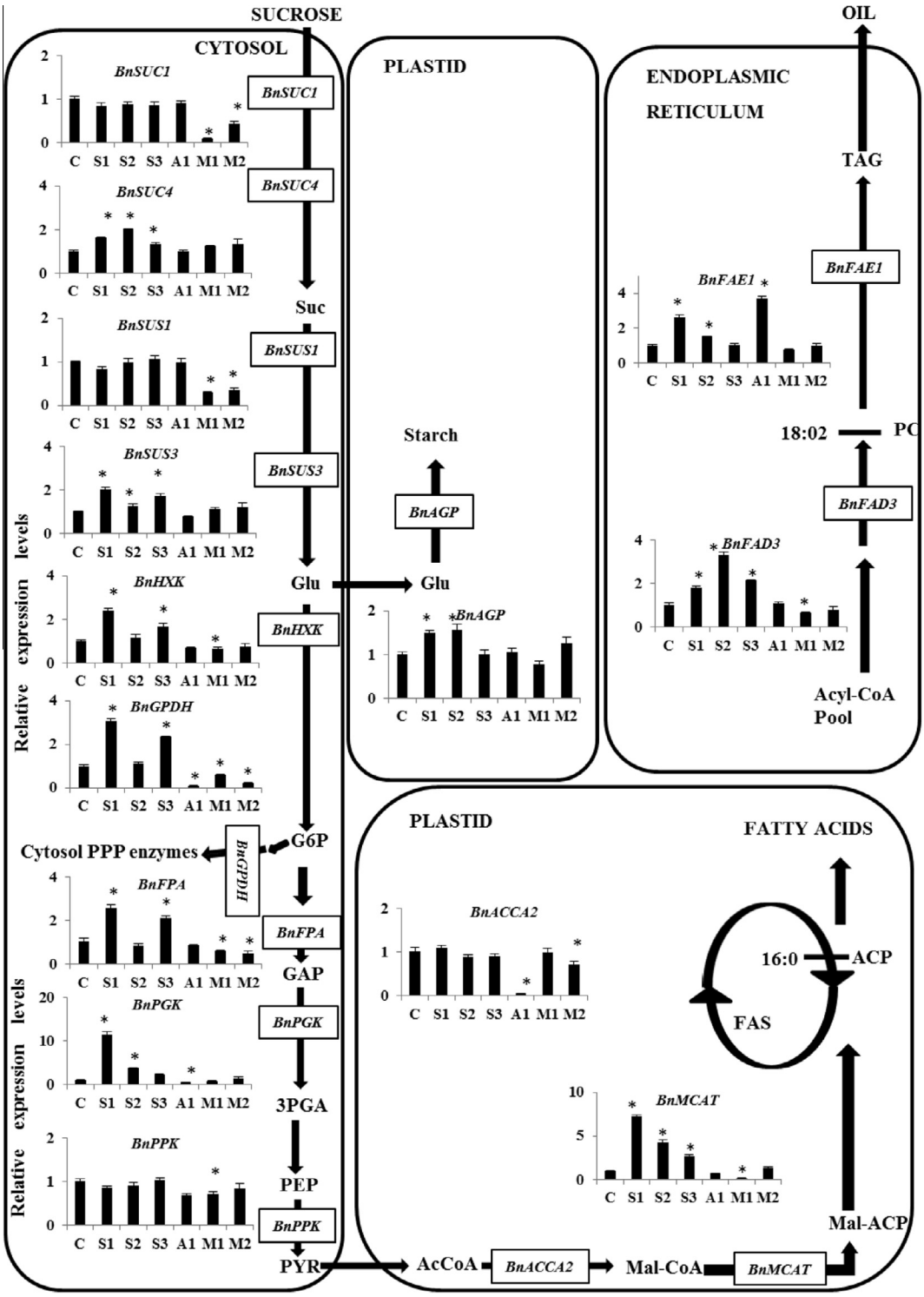

Figure 9 Expression analysis by quantitative (q)RT-PCR of genes involved in the regulatory pathways leading to FA synthesis in *BnLEC1* transgenic and *BnFUS3* tilling mutants MDEs at 21 days. *SUC1* (*Sucrose Transporter1*), *SUC4* (*Sucrose Transporter4*), *SUS1* (*Sucrose Synthase1*), *SUS3* (*Sucrose Synthase3*), *AGP* (*ADP-Glucose Phosphorylase*). *FPA* (*Fructose Bisphosphate Aldolase*), *PGK* (*Phosphoglycerate Kinase*), *GPDH* (*Glyceraldehyde-3- Phosphate Dehydrogenase*), *HXK* (*Hexose Kinase*), *PPK* (*Pyrophosphatase-Dependent Phosphofructokinase*). *ACCA2* (*Subunit A of Acetyl-CoA Carboxylase*), *FAD3* (*ω-3 FA Desaturase*), *FAE1* (*FA Elongation1*), *MCAT* (*Malonyl-CoA:ACP Transacylase*). Data presented are mean value ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective wild type value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1, S3), *BnLEC1* down-regulator (A1), *BnFUS3* tilling mutants (M1, M2).

reducing embryo yield and regeneration frequency. Based on these results it is suggested that Brassica microspore-derived embryogenesis is a suitable *in vitro* model for oil research, in particular to further examine the role of *BnLEC1* and *BnFUS3*, although the reduction in embryo number and quality can pose limitations to its use as a propagation tool to regenerate the transformed embryos.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jgeb.2016.05.002.

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